Alteration of neural tissue structure by expression of polysialic acid induced by viral delivery of PST polysialyltransferase

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The expression of polysialic acid (PSA) on neural cell adhesion molecule (NCAM) is known to attenuate cell–cell interactions. During neural development the widespread expression of PSA-NCAM creates permissive conditions for the migration of neuronal and glial precursors and the guidance and targeting of axons. NCAM polysialylation can occur via either of two specific sialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST), and the purpose of this study was to determine if retroviral delivery of either PST or STX could induce PSA expression in vivo and thereby alter tissue plasticity. Retroviruses expressing GFP-PST or GFP-STX were injected into embryonic retina, and development was evaluated by examining neuroepithelial structure, the expression of markers for specific cell types, cellular proliferation, and apoptosis. Chick retina was chosen because it down-regulates PSA early in its development and has a highly stereotyped program of morphogenesis. Retroviral expression of PST induced PSA expression in retina and resulted in severe but localized alterations in retinal morphogenesis, including an early disruption of radial glial cell morphology, highly disorganized retinal layers, and invasion of pigmented cells into the neural retina. In contrast, retroviral delivery of STX did not induce PSA expression or affect morphogenesis. These findings demonstrate that expression of PSA is sufficient to promote morphological alterations in a relatively nonplastic neural tissue.

Key words: plasticity/polysialic acid/polysialyltransferase/PST/STX

Introduction

During the evolution of vertebrates the variety of cell interactions and receptors expanded dramatically, particularly in the nervous system, and a unique mechanism appeared that provided for global regulation of interactions through steric hindrance of membrane–membrane apposition. This mechanism, using the regulated cell surface expression of the polysialic acid (PSA) carbohydrate attached to neural cell adhesion molecule (NCAM), promotes cell migration, axon guidance, and formation of terminal arbors in the brain (Rutishauser, 1998; Bruses and Rutishauser, 2001; Kiss et al., 2001). In these contexts it is likely that PSA acts purely as a permissive agent, in that it allows change to occur through an attenuation of existing cell interactions but does not specify the nature of that change. In most cases PSA is down-regulated soon after a mature tissue has formed. However, PSA expression persists in restricted regions of the central nervous system and confers structural plasticity that helps these tissues respond appropriately to physiological inputs (Seki and Arai, 1993; Kiss and Rougon, 1997; Durbec and Cremer, 2001; Kiss and Muller, 2001).

The primary goal of the present study was to demonstrate the ability to misexpress PSA in vivo by viral delivery of cDNAs encoding either the ST8Sia IV (PST) or ST8Sia II (STX) polysialyltransferase and to determine if PSA mis-expression is sufficient to alter the structure of a central nervous system tissue. PST and STX have previously been shown in vitro to synthesize PSA chains specifically on the core carbohydrate of NCAM (Eckhardt et al., 1995; Nakayama et al., 1995, 1998; Angata and Fukuda, 2003). A further motivation for these studies is the possibility that PSA expression might provide a means to promote a permissive environment for repair mechanisms in adult tissues that no longer express PSA (Kiss et al., 2001).

The embryonic retina was chosen as a test system because it has a highly stereotyped program of morphogenesis and loses its PSA at an early stage in its development (Schloshauer et al., 1984). Because PSA acts as a permissive factor for tissue remodeling, its action requires a context in which other factors provide the driving force for change. We reasoned that the continuing process of development in the retina would serve this purpose, so that the introduction of ectopic PSA would distort the behavior of the cells, resulting in observable changes in retinal structure. Using retroviral delivery of PST, it has been possible to misexpress PSA and dramatically modify the fundamental structure of the retinal epithelia. The nature of these defects suggests that the primary effect of ectopic PSA is to alter interactions among cells within and at the edges of epithelial margins, consistent with a loss of tissue stability.

Results

Analysis of PSA-NCAM in the developing retina

As explained in the Introduction, one of the reasons that the developing chick retina was chosen for these studies is its relatively low level of NCAM polysialylation. As shown in Figure 1A, some PSA immunoreactivity is present...
These characteristics are critical in that the enhancement of PSA expression by PST and STX requires the presence of NCAM that is capable of further modification by sialyltransferase. Though nearly all NCAM expressed in the tectum carries PSA, as evidenced by the large increase in the 140-kDa NCAM band after removal of PSA with endo-neuraminidase (endo N), there is a significant amount of the 140-kDa band in retina prior to endo N treatment (Figure 1B). Furthermore, the length of the PSA chains in retina, as represented by an apparent size for the PSA-NCAM of 140–200 kDa, is less than for tectal PSA-NCAM, which extends from 200 kDa to beyond 250 kDa.

**Induced expression of PSA-NCAM in CHO cells transfected with CMV-GFP-PST or CMV-GFP-STX**

The next step in the study was to evaluate the nature of the PSA produced by transfection with PST and STX. Again, it was important to assess not only the extent of NCAM polysialylation but also the nature of the chains produced, because excessively long chains could produce artifacts unrelated to PSA’s physiological function. Furthermore, it was necessary to compare PSA produced by PST and STX, because these enzymes exhibit differences in their ability to polysialylate NCAM (Angata et al., 1998, 2002; Angata and Fukuda, 2003). Chinese hamster ovary (CHO) cells were transiently transfected with NCAM180 cDNA and plasmids encoding GFP-PST or GFP-STX (Figure 2). Green fluorescent protein (GFP) fluorescence indicating expression of GFP-PST or GFP-STX is shown in Figures 2C and 2D, and these cells were stained using anti-NCAM (Figures 2A and 2B) and anti-PSA antibodies (Figures 2E and 2F). GFP-STX and GFP-PST were detected predominantly in the juxtanuclear Golgi region as well as at the cell surface, in agreement with previous reports using epitope-tagged STX and PST (Close and Colley, 1998). As expected, PSA staining was only detected in GFP-positive cells that are stained with anti-NCAM. Expression of GFP-PST resulted in higher levels of staining for PSA than expression of GFP-STX (compare Figures 2E and 2F). Treatment of the transfected cells with endo N prior to addition of antibodies abolished all PSA staining (unpublished data). Similar results were also obtained using COS cells.

To further evaluate the PSA-NCAM produced by GFP-STX and GFP-PST, anti-NCAM and anti-PSA immunoblots were carried out using transfected CHO cells (Figures 3A and 3B). The expression of PSA by GFP-PST was confirmed in Figure 3A as shown by the presence of a diffuse high molecular weight band in lane 2 that could be converted to a sharp band after endo N treatment. This broad band was not observed in cells transfected with the NCAM180 cDNA alone. Transfection of a plasmid encoding NCAM180 and a nontagged form of human PST resulted in a similar extent of NCAM polysialylation as with the GFP-tagged form. The size range of the PSA-NCAM produced by GFP-PST (140–250 kDa) indicates that it lies in between that naturally observed for the retina and tectum (see Figure 1B). Anti-PSA immunoblots comparing PSA produced by GFP-PST and GFP-STX showed that PST produced longer PSA polymers than STX (Figure 3B). The size of PSA synthesized by STX ran primarily throughout the retina at embryonic days (E) 4.5 and 8, with highest levels detected in the developing optic fiber layer (OFL). At later stages of development (E10–12), PSA expression is more restricted to the OFL and the inner plexiform layer (IPL), whereas expression is reduced in the outer retina. PSA staining was not detected in the pigmented epithelium at any developmental age. NCAM is broadly expressed throughout the retina at all developmental stages (unpublished data).

The degree of NCAM polysialylation in retina is lower and the polymer length of PSA is shorter (Schlosshauer et al., 1984), as compared to developing brain tissue.

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**Fig. 1.** Expression of PSA during chick retinal development. (A) Sections of retina stained with anti-PSA antibodies show abundant PSA expression throughout the retina at E4.5 and E8. At E8, E10, and E12 PSA staining is strongest in RGC axons in the OFL seen at top of images. At E10 and E12 PSA expression is progressively more restricted to the IPL and the OFL and decreased in the outer portions of the retina. PSA staining is detected at very low levels in the outer retina at E12. PSA was not detected in the pigmented epithelium (PE, indicated by asterisks) at any developmental stage. Scale bar equals 50 μm. (B) Western blot analysis of PSA-NCAM in the retina and tectum demonstrates that a significant fraction of NCAM expressed in the retina is nonpolysialylated (lane 1), whereas the bulk of NCAM is highly polysialyated in tectum (lane 3). The size range of PSA-NCAM in the tectum is also greater than that in the retina (compare lanes 1 and 3). Treatment of samples with endo N in lanes 2 and 4 converted the diffuse high molecular weight PSA-NCAM to a sharp NCAM 140 band.

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between 200–250 kDa (lane 3), whereas PSA synthesized by PST ran above 250 kDa (lane 5) and is more similar to that naturally found in the tectum (lane 7).

**Misexpression of PSA by retroviral delivery of GFP-PST disrupts normal histogenesis of the retina**

The effect of PSA misexpression on the development of the chick retina was studied by infecting embryos at Hamburger-Hamilton stage 10–12 with a replication competent avian leukemia virus GFP-PST (RCAS-GFP-PST) retroviral construct (Figure 4). The extent of infection was monitored by examination of GFP fluorescence and staining retinal sections with an antibody that recognizes viral gag matrix protein (Potts et al., 1987). RCAS-GFP retrovirus-infected cells and their progeny formed dense columns of cells spanning the thickness of the retina (Figure 4B). With RCAS-GFP, detection of GFP fluorescence coincided with the immunohistochemical localization of gag protein (unpublished data), and the expression of PSA was the same in infected and uninfected regions of the retina (Figure 4C). RCAS-GFP also had no effect on the retina at E8 in terms of the overall histological structure of the tissue (Figure 4A).

In embryos infected with RCAS-GFP-PST, ectopic PSA was expressed in regions where GFP-PST was detected (Figures 4E and F, H and I, K and L). In contrast to GFP-PST, retroviral expression of GFP-STX did not produce ectopic PSA in the retina (Figures 4Q–R). At E8–14, misexpression of PSA in the retina produced localized changes in retina histology. In each case the tissue in the infected region had a disorganized appearance (mild in D,
Retroviral expression of GFP-PST but not GFP-STX produces ectopic PSA and disrupts retinal morphology. Embryos were infected with RCAS-GFP (A–C), RCAS-GFP-PST (D–L), or RCAS-GFP-STX (P–R). Retroviral expression of GFP does not alter the expression of PSA at E8 (C). Misexpression of GFP-PST is associated with increased PSA expression at E8 (F and I). In a more widely infected region of E8 retina, GFP-PST expression is associated with an increase in PSA throughout the retina (I) and a more severely disrupted retinal morphology (G). At E14, the presence of ectopic PSA (L) is associated with highly disorganized retinal structure and the invasion of pigmented epithelial cells into the neural retina (J). Coinjection of RCAS-GFP-PST and endo N to remove endogenous and GFP-PST derived PSA rescues effects produced by PSA misexpression in the retina (M–O). At E10, endo N completely removes PSA throughout the retina (O). Despite the widespread expression of GFP-PST (N), no effects on retinal morphogenesis were observed (M). Infection with GFP-STX was expressed (Q), this did not result in ectopic PSA (R) or changes in tissue architecture (P). Scale bar equals 50 μm.

Fig. 4. Retroviral expression of GFP-PST but not GFP-STX, produces ectopic PSA and disrupts retinal morphology. Embryos were infected with RCAS-GFP (A–C), RCAS-GFP-PST (D–L), or RCAS-GFP-STX (P–R). Retroviral expression of GFP does not alter the expression of PSA at E8 (C). Misexpression of GFP-PST is associated with increased PSA expression at E8 (F and I). In a more widely infected region of E8 retina, GFP-PST expression is associated with an increase in PSA throughout the retina (I) and a more severely disrupted retinal morphology (G). At E14, the presence of ectopic PSA (L) is associated with highly disorganized retinal structure and the invasion of pigmented epithelial cells into the neural retina (J). Coinjection of RCAS-GFP-PST and endo N to remove endogenous and GFP-PST derived PSA rescues effects produced by PSA misexpression in the retina (M–O). At E10, endo N completely removes PSA throughout the retina (O). Despite the widespread expression of GFP-PST (N), no effects on retinal morphogenesis were observed (M). Infection with GFP-STX was expressed (Q), this did not result in ectopic PSA (R) or changes in tissue architecture (P). Scale bar equals 50 μm.
To characterize the cellular events underlying these morphological defects, the structure of retinal neuroepithelial cells and Muller glia was monitored by staining with anti-vimentin antibodies. The magnifications used illustrate the overall radial organization of these cells in the retina. In regions infected with RCAS-GFP-PST and displaying ectopic PSA, the normally uniform and spindle-shaped vimentin-positive cells were already slightly altered as early as E4.5 (Figures 5A–C). By E6.5 this subtle alteration has translated into more evident defects in retinal histogenesis (Figure 5E), which become more severe at later stages (Figures 5H, K). What is evident in all these defects is a loss of the ability of the vimentin-positive cells to span the epithelium. Having lost their association with the epithelial margin, these cells became shorter and thicker, with dense staining for vimentin.

Once the basic structure of the neuroepithelium had been compromised by the ectopic PSA, it would be expected that other retinal cell types would no longer be capable of carrying out well-organized histogenesis. To examine this possibility, the development of retinal ganglion cells (RGCs) was monitored using an antineurofilament antibody to label their axons and the islet-1 monoclonal antibody to label RGC soma (Figure 6). RGC axons in infected regions were not longer confined to the optic fiber layer (compare Figures 6A and B). In addition, staining with islet-1 showed that RGCs in an infected region were scattered throughout the inner and outer nuclear layers (Figure 6D). Therefore it is likely that some of the ectopic RGC axons shown in Figure 6B originated deep within the retina rather than at the vitreal margin.

### Effects of PSA misexpression on cell death and cell proliferation

Although the observations indicate that PSA misexpression disrupts retinal development by altering the basic architecture of the retina, it is also possible that the defects could be a consequence of effects on cell division and/or death. However, staining for mitotic cells with anti-BrdU showed no increase in cell proliferation activity in infected regions as compared to adjacent uninfected regions at E8 (Figures 7A and B). Embryos infected with RCAS-GFP as a control also showed no change in the number of BrdU-labeled cells (unpublished data).

The effect of PSA misexpression on programmed cell death was monitored using the TUNNEL assay (Figure 8). In the retina, only a modest level of apoptosis occurs prior to E10 (Rager, 1980; Mey and Thanos, 2000). From E10–14, up to 40–45% of RGCs die after their axons have invaded the optic tectum. In retinas infected with RCAS-GFP-PST, TUNNEL labeling at E4.5 showed no evidence of enhanced cell death in PST-positive regions (unpublished data). At E5.5 infected regions occasionally contained a few TUNNEL-positive cells (Figures 8A–C). However, close examination of individual cells indicated that only a small subset of PSA-positive cells were TUNNEL-positive. At this stage, TUNNEL-positive cells were not detected in uninfected regions except in the central-most retina (unpublished data). At E8, when naturally occurring cell death is very low (Fradet et al., 1997), patches of TUNNEL-labeled cells were found in regions exhibiting morphological defects induced by PSA misexpression.

### Table I. Retinal phenotypes are rescued by coinjection of endo N with RCAS-GFP-PST

<table>
<thead>
<tr>
<th>Injection</th>
<th>Severe</th>
<th>Mild</th>
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<td>16</td>
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<td>16</td>
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<td>Group 2 PST + endo N</td>
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The results of two independent experiments (groups 1 and 2) show the effects on retinal development of RCAS-GFP-PST injection alone or coinjection of RCAS-GFP-PST with endo N. In each experimental group, three embryos injected with PST-virus alone or with PST-virus and endo N were analyzed at E10. PSA immunohistochemistry was carried out to ensure complete removal of PSA by endo N. For each embryo, 20 GFP-positive regions were identified and examined using light microscopy to score retinal phenotypes. The effects of PSA misexpression were scored as severe if cells from the retinal pigmented epithelium had invaded the neural retina (as in Figure 4J) or if the retina was markedly thinner in the GFP-positive region. Mild defects were defined as changes in the histological organization of the retina as seen by the disorganized appearance of cells and layers compared to a surrounding uninfected region (as seen in Figure 4G). Nonspecific defects were scored based on the presence of sectioning artifacts such as cuts, tears, or folds in the tissue or a separation of the retinal pigmented epithelium from the neural retina.

Severe in G and J) as compared to an adjacent region of uninfected retina. In J the defects include the adjacent pigmented epithelium, whose cells have invaded the neural epithelium. Thus the misexpression of PSA not only can alter the pattern of cells within a tissue structure but also can compromise the integrity of adjacent tissue layers. As might be expected, the expression of STX did not produce detectable distortions of retinal architecture (Figure 4P–R). In regions expressing GFP-PST, PSA expression was more widespread than the expression of GFP. This reflects the fact that GFP-PST accumulates in the Golgi whereas PSA is expressed on the cell surface, and cellular processes can extend far from cell bodies where GFP-PST is localized.

To confirm that these defects specifically reflect cell surface PSA misexpression, endo N was injected along with the RCAS-GFP-PST, so that any PSA expression (ectopic or endogenous) is suppressed. The action of endo N under these conditions is complete and long lasting (Landmesser et al., 1990; Tang et al., 1992; Yin et al., 1995), so that no cell surface PSA can be detected throughout the period of the experiment (Figure 4O). Though removal of endogenous PSA by endo N did not by itself alter retinal development, in infected regions it did prevent defects caused by misexpression of PST (Figures 4M, N). This rescue by endo N is scored quantitatively in Table I, showing that expression of GFP-PST in the presence of endo N resulted in mild defects in only 3/120 GFP-positive regions, whereas expression of GFP-PST without endo N resulted in severe or mild defects in 117/120 GFP-positive regions.

**PSA misexpression alters the morphology and organization of radial neuroepithelial and Muller glial cells**

To characterize the cellular events underlying these morphological defects, the structure of retinal neuroepithelial...
(Figures 8D–F). At E12 a large number of TUNNEL-positive cells were detected only in regions expressing GFP-PST (Figures 8G–I). TUNNEL-positive cells were particularly abundant in the outer retina where programmed cell death is not normally detected at this stage (Frade et al., 1997).

Altogether, these results demonstrate that the increase in apoptosis associated with ectopic PSA occurs later than the induced structural defects, does not directly reflect PST expression by a particular cell, and most likely represents a secondary consequence of altered trophic interactions. The increase in cell death may also be compounded by the presence of RGCs at ectopic locations (Figure 7D) as it has been demonstrated that RGCs can regulate the killing of other RGCs by secreting nerve growth factor (Gonzalez-Hoyuela et al., 2001).

Discussion

These studies demonstrate that retroviral expression of the sialyltransferase PST can direct the synthesis of ectopic PSA-NCAM in a central nervous system tissue, the chick retina. PSA misexpression severely disrupts the normal transition of the retina from a homogeneous neuroepithelium to a highly organized layered structure. Although both the PST and STX polysialyltransferases are able to polysialylate NCAM in vitro, only PST was able to induce detectable PSA expression or affect retinal development in vivo.

NCAM polysialylation by PST and STX
in vitro and in vivo

The first issue raised by these findings is whether the observed biological defects might be an artifact of a grossly...
nonphysiological level or state of the PSA polymer. That is, the massive overexpression (amount or length) of any such charged polymer might nonspecifically disrupt a cell’s ability to behave normally or even survive within a tissue. 

In vitro experiments using PST and STX have established that either enzyme is capable of polysialylating NCAM (Eckhardt et al., 1995; Kojima et al., 1995; Nakayama et al., 1995; Scheidegger et al., 1995; Nakayama and Fukuda, 1996), and in our study both GFP-STX and GFP-PST produced cell surface PSA-NCAM in CHO, COS, and Neuro2a cells (Figure 2 and unpublished data). Neither the amount of PSA nor the length of the polymers induced appeared to lie outside the observed physiological range, in that all of the NCAM in normal embryonic brain appears to carry PSA and the natural polymer length (as judged by electrophoretic mobility) was not exceeded in the transfection studies. Furthermore, the presence of the GFP moiety did not appear to affect the enzymatic capacity of PST or STX as the GFP-tagged enzymes produced PSA of similar quality as untagged PST. Although it is possible that evaluation of these parameters in cell lines is not entirely representative of polysialylation in intact retina (see discussion of STX), it is also reassuring that the level of PST-induced ectopic PSA in the retina as judged by immunofluorescence was similar to that for endogenous PSA in retina and less than that found naturally in the optic tectum.

The present studies confirm earlier reports that PSA-NCAM produced in vitro by PST contains more sialic acid residues than those obtained with STX (Angata et al., 1998, 2002; Kitazume-Kawaguchi et al., 2001). An even more striking difference was seen in vivo, where retroviral expression of PST produced ectopic PSA but STX expression had no detectable effect on PSA levels. This difference may be related to the polysialylation status of NCAM in developing retina, where NCAM is relatively PSA-deficient both in terms of the presence of nonpolysialylated NCAM and the length of PSA polymers. That poorly polysialylated NCAM may be a better substrate for PST than STX is consistent with reports that PST can both increase the length of PSA chains synthesized by STX and add PSA to nonpolysialylated antennas on NCAM N-glycans (Angata et al., 2002; Angata and Fukuda, 2003). The expression of PSA by GFP-STX in CHO cells together with the inability of GFP-STX to produce ectopic PSA in retina may suggest that more stringent regulatory mechanisms operate to control NCAM polysialylation in vivo than those operating in vitro.

Effects of ectopic PSA on retinal development
One goal of this study was to establish that PSA is not only necessary for promoting a variety of developmental changes in tissue structure (see introduction) but also sufficient to promote such changes. The PSA gain-of-function approach used here supports this capability in the context of the developing retina. However, it should be noted that if PSA creates permissive conditions for change, then its effects should be detected only when the cells in a tissue are actively attempting to alter their position or shape. In fact, it has been shown that the removal of naturally expressed PSA does not have a detectable effect during periods when a
tissue is not undergoing intrinsic reorganization of its architecture (Cremer et al., 1994; Yin et al., 1995; El Maarouf and Rutishauser, 2003).

The mechanism whereby PSA misexpression perturbs radial cell morphology and retinal histogenesis likely involves a loss of normal cell–cell or cell–matrix interactions between neuroepithelial cell endfeet and the retinal or pial basal lamina. The radial processes of neuroepithelial cells serve as guideposts for the normal migratory pathways of postmitotic retinal cell types (Rakic and Caviness, 1995) and are critical in both establishing retinal polarity and determining the pattern of retinal lamination and the formation of the optic nerve (Halfter, 1980; Mey and Thanos, 2000; Willbold et al., 2000; Halfter et al., 2001). In embryonic chick retina, where the basal lamina is transiently removed during development, the endfeet of neuroepithelial cells become irreversibly retracted, and the orderly migration of cell types, the development of retinal layers, and the growth of RGC axons are perturbed (Halfter, 1998; Halfter et al., 2001). Thus, ectopic PSA in radial cells may trigger altered cell–cell or cell–matrix interactions that severely disrupts radial cell morphology leading to the observed effects on retinal histogenesis. Ectopic PSA may also reduce the ability of postmitotic cells to interact with and migrate along processes of neuroepithelial cells.

In considering possible molecular targets for PSA in this system, it is interesting to note the role of cadherins in forming and maintaining epithelial structure (Gumbiner, 1996). Masai et al. (2003) demonstrate that zebrafish with mutations in N-cadherin have impaired organization and maintenance of adherens junctions in retinal neuroepithelial cells and are unable to organize their retinal neurons into correct layers. Such an association of cadherin function with the effects induced by PSA is also consistent with the fact that PSA has been shown to be a potent negative regulator of cadherin adhesive function (Fujimoto et al., 2001).

Just as up-regulation of PSA is valuable for promoting morphogenesis, so also is its down-regulation important for maintaining the structures that have been assembled. Expression of the polysialylated form of NCAM during an active phase of tissue development is typically followed by a progressive loss of PSA as more stable mature adult tissues are formed. For example, in the chick tectum, PSA-NCAM expression is down-regulated immediately following the innervation of retinal axons (Yamagata et al., 1995). Similarly, PSA expression is tightly regulated during...
synaptogenesis, being highly expressed on the surface of ciliary neurons prior to synapse formation and then progressively lost from points of synaptic contact (Bruses et al., 1995, 2002). Another example is the transient expression of PSA as myotubes separate from myotube clusters to form individual muscle fibers (Fredette et al., 1993). In the present study, the importance of this down-regulation to tissue stability is illustrated functionally by the fact that an abnormal persistence of PSA severely degrades the ability of the retina to maintain its overall epithelial integrity and thus the formation of organized layers.

Although the misexpression of PSA has detrimental effects in the developing retina, it could have beneficial effects in promoting tissue plasticity and repair in response to pathological insult. The fact that the brief period of nerve sprouting following nerve injury is functionally correlated with a transient up-regulation of PSA (Kiss et al., 2001), suggests that a more persistent expression might be useful. Moreover, the nature of the tissue perturbations found in the present studies suggest that PSA expression can be effective in disrupting interactions involving nonneuronal cellular elements, without affecting the ability of neurons to extend axons.

Materials and methods

Plasmid and viral constructs, viral production, and titering

The coding region of chicken ST8SiaIV/PST (Bruses and (Rutishauser, 1998; GenBank accession number AF008194) was polymerase chain reaction (PCR)-amplified using high-fidelity Pwo polymerase (Boehringer Mannheim, Mannheim, Germany). The 5′ PCR primer (5′-AGATATCCCAGGGAGACGTACATCCGGAGCGACGTCAAGAAG-3′) and 3′ PCR primer (5′-AGTGCTCCGGATTTGCTGTACACATTTCCC-3′) introduced BspE1 sites for insertion into pEGFP-C1 (Clontech, Palo Alto, CA) to generate pCMV-GFP-PST. Sequences encoding a glycine-rich stretch of six amino acids were contained in 5′ primers to insert a flexible linker region between GFP and PST. The fragment encoding GFP-PST was subcloned into NcoI/SmaI sites of the pCla12Nco helper plasmid and transferred to the ClaI site of the retroviral vector RCASBP(B). To prepare RCASBP(B)GFP, the pEGFP vector was digested with NcoI and NheI and ligated to NcoI/EcoR1 sites of the pCMV-NCAM180 vector used to express NCAM180 and PST from the same plasmid have been propagated and expanded over 10 days in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2% chick serum, by which time 100% of the cells were infected. When cells reached confluence after the last split, a minimal volume of low-serum media was added (DMEM with 2% FCS and 0.2% chick serum). After 24 h, media was removed and filtered through a 0.45-μm cellulose acetate filter. Media was replaced, and a second harvest was carried out 24 h later. Viral conditioned media was centrifuged for 3 h at 4°C in a Beckman SW28 rotor at 21,000 rpm. Viral pellets were resuspended in Opti-Mem on ice (Life Technologies, Cergy Pontoise, France) using 0.05 times the original volume. Retroviral titers were determined according to published protocols by immunocytochemistry with AMV3C2 anti-gag antibody (Morgan and Fekete, 1996; Developmental Studies Hybridoma Bank, Iowa City, IA). Immunocytochemistry for titering was carried out with DF-1 cells in chamber slides as described shortly, and titers for all virus preparations were in the range of 3 × 10^6 to 5 × 10^6 infectious units/ml.

Expression of NCAM, STX, and PST in CHO Cells

CHO cells obtained from the American Type Tissue Collection were transiently transfected at 90% confluency with pCMV-GFP-PST and pCMV-NCAM180, pCM-GFP-STX and pCMV-NCAM180, or pCMV-NCAM180 alone using LipofectAMINE2000 according to the manufacturers instructions. Cells were grown in 60 mm dishes or in eight-well chamber slides (Tissue-Tek, Naperville, IL) in DMEM supplemented with 10% FCS. Thirty-six hours after lipofection cells were harvested in extraction buffer containing 20 mM Tris–HCl, 150 mM NaCl, 2 mM ethylenediamine tetra-acetic acid, and 0.8% Nonidet P-40 containing a protease inhibitor mixture (Boehringer Mannheim). Suspensions were sonicated on ice three times for 10 s and incubated at 4°C for 1 h. Samples were then centrifuged at 15,000 × g for 10 min, and the protein concentration in the supernatant was determined using the BCA method (Pierce, Rockford, IL).

Immunocytochemistry for PSA and NCAM in CHO Cells

CHO cells were transfected as described in eight-well chamber slides. Thirty-six hours after transfection cells were fixed for 15 min at room temperature in phosphate buffered saline (PBS)/4% paraformaldehyde and rinsed 4 times 10 min in PBS. Blocking was carried out for 60 min at room temperature in PBS-T containing 0.05% Triton X-100 and 1% ultra-pure IgG-free bovine serum albumin (Jackson Immunoresearch, West Grove, PA). Cells were then incubated overnight at 4°C with 1:1500 dilutions of the monoclonal antibodies 5e that recognizes chick NCAM (Watanabe et al., 1986) and 5a5 that recognizes PSA (Acheson et al., 1991). Cells were washed three times 10 min in PBS-T and incubated for 1 h in blocking buffer with the appropriate Cy5- (1:400) or Cy3-conjugated (1:800) antibodies (Jackson Immunoresearch). After rinsing four times 15 min, slides were coverslipped with mowiol containing 0.01% triethylene-diamine (Sigma-Aldrich, Deisenhofen, Germany). Staining for NCAM and PSA was abolished in control experiments where either primary antibody was omitted.

Induction of polysialic acid in neural tissue by PST

Cilia of distal with basal domain y 0.5-μm length were stained with tetracaine acetic acid and 0.8% Nonidet P-40 containing a protease inhibitor mixture (Boehringer Mannheim). Suspensions were sonicated on ice three times for 10 s and incubated at 4°C for 1 h. Samples were then centrifuged at 15,000 × g for 10 min, and the protein concentration in the supernatant was determined using the BCA method (Pierce, Rockford, IL).

Immunocytochemistry for PSA and NCAM in CHO Cells

CHO cells were transfected as described in eight-well chamber slides. Thirty-six hours after transfection cells were fixed for 15 min at room temperature in phosphate buffered saline (PBS)/4% paraformaldehyde and rinsed 4 times 10 min in PBS. Blocking was carried out for 60 min at room temperature in PBS-T containing 0.05% Triton X-100 and 1% ultra-pure IgG-free bovine serum albumin (Jackson Immunoresearch, West Grove, PA). Cells were then incubated overnight at 4°C with 1:1500 dilutions of the monoclonal antibodies 5e that recognizes chick NCAM (Watanabe et al., 1986) and 5a5 that recognizes PSA (Acheson et al., 1991). Cells were washed three times 10 min in PBS-T and incubated for 1 h in blocking buffer with the appropriate Cy5- (1:400) or Cy3-conjugated (1:800) antibodies (Jackson Immunoresearch). After rinsing four times 15 min, slides were coverslipped with mowiol containing 0.01% triethylene-diamine (Sigma-Aldrich, Deisenhofen, Germany). Staining for NCAM and PSA was abolished in control experiments where either primary antibody was omitted.
Western blotting
Protein extracts from chick retina and tectum were prepared by dissecting tissues in cold oxygenated Tyrode’s solution (134 mM NaCl, 3 mM KCl, 20.5 mM NaHCO₃, 3 mM CaCl₂, 1 mM MgCl₂, and 12 mM glucose, pH 7.2). Tissues were mechanically dissociated in extraction buffer using a fire-polished Pasteur pipette, then proteins were extracted as described. Equal amounts of protein were divided into aliquots and in some cases endo N, isolated with RCAS(B)GFP-PST with endo N and three embryos for 1 h at 37°C. Samples were heated at 65°C for 20 min and loaded on 6% sodium dodeyl sulfate-polyacrylamide gel electrophoresis gels using a Bio-Rad minigel system (BioRad, Munich, Germany). Proteins were transferred by electrophoresis onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h in TBS-T (0.1% Tween 20) containing 5% dry milk (BioRad), then incubated overnight at 4°C in a 1:1500 dilution of anti-NCAM antibody. Blots were incubated with peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) diluted 1:5000 in TBS-T. After incubation for 1 h at room temperature, membranes were washed four times for 5 min with TBS-T. Detection was carried out using the ECL method according to the manufacturers instructions (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Viral infection and coinjection of virus with endo N
Fertile white leghorn eggs, subtype 0 (Spafus, North Franklin, CT), were incubated at 38°C in a 100% humidified incubator. At 36 h embryos were windowed and staged according to Hamburger and Hamilton (1951). Embryos at Hamburger and Hilton stage 10–12 were used for injections. Approximately 100 nl of retroviral concentrates containing 0.02% fast green and 80 μl/ml of polybrene (Sigma-Aldrich) was pressure injected into the right optic vesicle using a micromanipulator (Parker Kannah Corp, Fairfield, NJ) attached to a micromanipulator (Narishige, East Meadow, NY). The solution was injected until it filled the telencephalon and both optic vesicles. Embryos were sealed with paraffilm and incubated until the desired stage. In preliminary experiments using RCAS(B)GFP, this method was found to provide a high level of infection in the retina as shown by staining with anti-gag antibody as well as by monitoring GFP expression. Injection at Hamburger and Hilton stage 10–12 with RCAS(B)GFP-PST or RCAS(B)GFP-STX typically resulted in infection of 40–50% of the retina, and infection with RCAS(B)GFP produced slightly higher rates of infection. In all RCAS injection experiments, representative sections were stained with anti-gag to assess the extent of infection. In additional sets of control experiments, endo N was coinjected together with RCAS(B)GFP-PST. Equal volumes of either PBS or endo N was added to viral concentrates and injected as described. Three embryos injected with RCAS(B)GFP-PST with endo N and three embryos injected with virus alone were analyzed from each of two independent experiments. Representative sections were stained with anti-PSA and anti-GAG antibodies to ensure complete removal of PSA by endo N and to access the extent of infection. The effects of GFP-PST expression in the presence or absence of endo N were then scored as described in Table I.

Immunohistochemistry and confocal microscopy
Chick eyes were dissected from white leghorn embryos (Spafus) of different ages in cold-oxygenated Tyrode’s solution and then fixed in 4% paraformaldehyde-PBS at 4°C. Eyes were then rinsed extensively in PBS with six changes over 4 h and placed in 20% sucrose overnight at 4°C for cryoprotection. The tissue was then embedded in optimal cutting temperature compound (Tissue-Tek) and sectioned at 16 μm using a cryostat. Tissue sections were air-dried and stored at −80°C. For immunohistochemistry on retinal sections, slides were incubated in 100 mM glycine in PBS for 10 min followed by 1 h in blocking solution (PBS containing 1% bovine serum albumin and 0.05% Triton X-100). Primary antibodies diluted in blocking buffer were incubated overnight at 4°C. NCAM distribution was visualized using the 5e antibody (1:1500), and PSA was detected with the 5A5 antibody (1:1500). Other antibodies used in this study (obtained from the Developmental Studies Hybridoma Bank) are anti-NF-M to label retinal axons (clone 4H6, 1:50), anti-vimentin to label glial cells (clone H5, 1:100), and islet-1 to label RGCs (clone 39.4D5, 1:100). Sections were then rinsed twice with PBS and incubated for 1 h at room temperature in blocking buffer and the appropriate cy3- or cy5-conjugated secondary antibodies. Single confocal scans were obtained using a Zeiss LSM 510 confocal microscope.

Analysis of cell division and death
Fifty microliters of BrdU solution (50 μg/ml; Amersham, Piscataway, NY) was placed onto E8 embryos that had been injected with RCAS(B)GFP-PST. Embryos were then incubated for 4 h at 38°C. Eyes were dissected and processed for immunohistochemistry as described, and anti-BrdU immunohistochemistry was carried out according to the manufacturer’s instructions (Amersham). To visualize cells undergoing cell death in the retina, the in situ Cell Death Detection Kit, TMR Red was used according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN).

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Abbreviations
CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle medium; endo N, endoneuraminidase; FCS, fetal calf serum; GFP, green fluorescent protein; IPL, inner plexiform layer; NCAM, neural cell adhesion molecule; OFL, optic fiber layer; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PSA, polysialic acid;
PST, ST8Sia IV; RCAS, replication competent avian leukemia virus; RGC, retinal ganglion cell; STX, ST8Sia II.

References


